STUDIES IN INFLUENZA AND PNEUMONIA

STUDY X. THE IMMUNOLOGIC PROPERTIES OF THE GREEN-PRODUCING STREPTOCOCCI FROM INFLUENZA

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In a previous report it was shown that most of the green-producing streptococci isolated so constantly in influenza were agglutinated specifically by a monovalent immune horse serum; that highly agglutinable strains absorb the agglutinins for the other strains; and that during convalescence in influenza the serum of patients acquires the power to agglutinate many of the freshly isolated green-producing streptococci. Attention was directed also to the fact that these organisms possess well marked antigenic properties, the serum of persons developing specific agglutinating power after injections of a mixed vaccine.

In this study I shall detail further results obtained by subjecting numerous strains of the green-producing streptococcus from influenza to the action of various immune serums, especially to the monovalent serum prepared with one of the strains of the green-producing streptococcus. The monovalent serum was prepared by injecting a large horse (horse 15) with increasing doses of one strain of green-producing streptococcus isolated from the blood after death in a case of influenza and influenzal pneumonia. The symptoms and findings in this case, reported elsewhere, were typical. The thorax was expanded and immobile, the patient expectorated a large amount of bloody, frothy fluid. The lung after death was voluminous, extremely wet with a dark colored bloody fluid and the seat of numerous coalescing areas of lobular pneumonia.

The strain as isolated from a single colony from the blood and after one animal passage was put aside on blood-agar slants and in deep tubes of dextrose-brain broth. Both of these produced typical, rather moist, spreading, greenish colonies on blood-agar plates, both fermented inulin, but they were not bile soluble. Cultures for immunization of the horse were made from the stock cultures in bottles of glucose broth containing 150 c c each. These were incubated over night, or until heavy growth had occurred, centrifuged and the sediment suspended in salt solution so that 1 c c of the sediment represented

the growth from 15 c c of the broth. Control blood-agar plate cultures were made of the material inoculated in the bottle as well as of the growth injected into the horse. The dense bacterial suspension was used for intravenous immunizations. The first injection, made Jan. 9, 1919, consisted of 6 c c of the suspension or the growth from 90 c c of broth. The injections were given on three successive days in each week. The first six injections consisted of the heat killed bacteria (60 centigrade for thirty minutes). After that live cultures were injected. The dose by March 3, when 3 liters of blood were withdrawn, had been increased to 50 c c of the suspension or the growth from 750 c c of the glucose broth culture. The injections were continued and the dose gradually increased until April 4, when 14 liters of blood were withdrawn. The horse was given a rest for ten days, and the injections were resumed, but owing to marked reactions and loss of weight the dose had to be diminished and finally was discontinued, April 16. In spite of the fact that no more injections were given the horse continued to lose in weight and strength, and June 4 it was unable to get up, and was bled to death under ether.

The serums obtained before the injections were begun, and on March 3, April 4 and June 4 after immunization, were titrated against freshly isolated strains of the green-producing streptococcus. It was found that the upper limit of agglutinating power of the serum obtained before the injections were begun was about 1 to 10; the serum obtained March 3, about 1 to 500; April 4, 1 to 1,000 to 1 to 10,000; and June 4, 1 to 500 or 1 to 1,000. The serum of the highest titer obtained April 4 was mostly used in the agglutination experiments herein reported.

The antihemolytic streptococcus serum (horse 9) was prepared by repeated injections with four strains of highly virulent hemolytic streptococci from cases of severe ascending infections and cases of cellulitis. The injections were given between December 18, 1917, and July 1, 1918. The serum during this time had acquired marked agglutinating power over the strains injected. It should be emphasized that all of these strains were isolated before the epidemic of influenza occurred.

The pneumococcus immune serums were obtained from Dr. Rufus I. Cole, of the Hospital of the Rockefeller Institute for Medical Research, and from Dr. Augustus B. Wadsworth, of the New York State Department of Health. These were titrated against known strains of type pneumococci and were found to possess marked and specific agglutinating power.

METHODS

The freshly obtained sputum was sent to the laboratory for cultures throughout the four epidemic waves of influenza in 1918 and 1919. The cultures and agglutination experiments were made and recorded without knowledge of the history of the patients. The diagnosis, days of onset and other data were ascertained later from the records. A series of preliminary experiments in which various dilutions of serums were used (from 1 to 10 to 1 to 10,000), showed that a final dilution of these serums of 1 to 20 had the widest range of usefulness. Accordingly, for routine work the mixture in each tube consisted of 0.2 c c of the various serums diluted 1 to 10 with salt solution, and 0.2 c c of the antigen. The antigen consisted, for the most part, of the dextrose-blood broth or dextrose-acacia-broth culture, or of a salt solution suspension of bacteria grown in these after they had been preserved in 50 per cent. glycerol for a variable length of time. In some instances the peritoneal washings of mice and guinea-pigs which had succumbed to injections of sputum or primary culture from sputum, were also used as antigen. During the first two waves dense suspensions of the green-producing streptococci from primary cultures of sputum or blood of animals dead from injection of sputum were filed away in 50 per cent. glycerol, so that 1 c c equaled the growth from 15 c c of the dextrose-acacia-broth culture. These were kept in the ice chest and diluted with 15 parts of salt solution at the time the agglutination tests were performed. The mixtures of serum and antigen were thoroughly shaken and incubated at 37 C. for from one to one and one-half hours and placed in the ice chest over night, before readings were taken. The amount of agglutination, as indicated in the tables, was recorded by from 1 to 4 plus signs, 1 plus indicating slight but definite agglutination, 2 plus decided clumping but with little sedimentation, 3 plus marked agglutination and sedimentation but with supernatant fluid, not entirely clear, and 4 plus complete agglutination with the bacteria packed quite solidly at the bottom and the supernatant fluid completely cleared.

"Specific" agglutination is the term applied to the serum which agglutinated a particular strain to a greater degree than any of the other serums.

In most cases only one or two samples of sputum were cultivated and the agglutination tests made with the bacteria thus obtained. In some instances the agglutination experiments were done with strains isolated from the sputum daily or on alternate days throughout the illness and with the strains isolated after death. In selected cases cul-

tures were made simultaneously of tonsil and of the throat or nasopharynx, and the strains isolated were subjected to the agglutinating action of the serums under identical conditions.

TABLE 1

AGGLUTINATION EXPERIMENTS WITH THE GREEN-PRODUCING STREPTOCOCCUS FROM INFLUENZA

Case or Strain	Date of Experiment	Date of Isolation	Source	Day of Disease	Antiserums					Controls	
					Pneumococcus			Streptococcus			
					I	II	III	Hemolytic Horse 9	Green Producing Horse 15	Normal Horse Serum	NaCl Solution
3218.2	3/ 9/19	3/ 7/19	Sputum	4	0	0	0	+	++	0	0
3218.2	3/ 9/19	3/ 7/19	Sputum	4	0	0	0	0	++	0	0
3225[2]	3/19/19	3/17/19	Sputum	3	0	0	0	++	+++	0	0
3225[2].5	6/17/19	3/17/19	Sputum	3	0	0	0	0	+	0	0
3266	3/25/19	3/24/19	Sputum	5	+	0	0	++	+++	+	0
3266[2].2	3/27/19	3/24/19	Sputum	5	0	0	0	0	0	0	0
3282	3/31/19	3/30/19	Sputum	2	0	+++	+++	+++	++++	0	0
3282	4/ 3/19	4/ 2/19	Sputum	5	0	++	+	++	+++	0	0
3332	4/ 7/19	4/ 6/19	Sputum	3	0	0	0	0	+++	0	0
3332.2	4/ 8/19	4/ 6/19	Sputum	3	0	0	0	0	++	0	0
3332	4/ 9/19	4/ 8/19	Tonsil	5	++	++	++	+++	+++	++	0
3332.2	4/10/19	4/ 8/19	Tonsil	5	0	++	0	0	+++	0	0
3332.6	11/18/19	4/ 8/19	Sputum	3	0	+	+	+	+	0	0
3334	4/ 7/19	4/ 6/19	Sputum	1	0	0	0	+++	++++	0	0
3334	4/ 8/19	4/ 7/19	Throat	2	0	0	++	+++	+++	0	0
3334.2	4/ 9/19	4/ 7/19	Throat	2	0	0	0	0	+++	0	0
3334.2	4/ 9/19	4/ 7/19	Throat	2	0	0	++	+	+++	+	0
3334.2	4/ 9/19	4/ 7/19	Throat	2	0	0	0	+++	++++	+	0
3334	4/10/19	4/ 9/19	Sputum	3	0	0	++	++	++	0	0
3334.6	11/11/19	4/ 9/19	Sputum	3	0	0	+	+++	+++	+	0
3365	4/14/19	4/13/19	Throat	3	0	0	++	0	+++	+	0
3365	4/14/19	4/13/19	Throat	3	0	0	0	0	++	0	0
3365.2	4/15/19	4/13/19	Throat	3	0	0	0	0	+++	+	0
3365	4/14/19	4/13/19	Sputum	3	0	0	0	0	+++	0	0
3365.2	4/14/19	4/13/19	Sputum	3	0	0	0	0	++	0	0
3365.3	4/16/19	4/13/19	Sputum	3	+	+	+	+	+++	0	0
3365.6	5/ 1/19	4/13/19	Sputum	3	0	0	0	0	++++	0	0
3365.6	5/ 1/19	4/13/19	Sputum	3	0	0	0	0	++++	0	0
3365.7	11/ 4/19	4/13/19	Sputum	3	0	0	0	+	+++	0	0
3366	4/14/19	4/10/19	Tonsil	2	0	0	0	0	0	0	0
3366	4/14/19	4/10/19	Throat	2	0	0	0	0	+	0	0
3366	4/14/19	4/10/19	Sputum	2	0	0	0	0	++	0	0
3366	4/14/19	4/12/19	Sputum	4	0	0	++	++	+++	+	0
3366.2	4/14/19	4/12/19	Sputum	4	+	+	+	+	+++	+	0
3366.3	6/17/19	4/12/19	Sputum	4	0	0	0	0	+++	0	0
3366.7	11/14/19	4/12/19	Sputum	4	0	0	0	0	++	0	0
3370	4/14/19	4/11/19	Sputum	2	0	0	0	0	++	0	0
3370.2	4/14/19	4/11/19	Sputum	2	0	0	0	0	++	0	0
3370.3	4/16/19	4/11/19	Sputum	2	0	0	0	+	++	0	0
3370.4	4/21/19	4/11/19	Sputum	2	0	0	0	0	++	0	0
3370.6	5/ 1/19	4/11/19	Sputum	2	0	0	0	0	+++	4	0
3370.7	11/22/19	4/11/19	Sputum	2	++	+	+	++	+++	++	0

RESULTS

In table 1 are summarized representative experiments indicating the results obtained with the green-producing streptococcus isolated from patients with influenza. In these and other experiments the following findings were noted:

1. The monovalent serum of horse 15 agglutinated specifically most of the strains isolated throughout short initial attacks of typical influenza (cases 3218, 3225 and 3332).

2. Specific agglutination occurred (often in duplicate) in the primary mass culture of sputum or throat swab and of material from animals dead from injection of sputum, and in the early subcultures of the green-producing streptococcus isolated from sputum, throat swab and animals injected with these strains (cases 3225, 3266, 3365 and 3366).

3. The immunologic condition of the green-producing streptococci, as manifested by their agglutinability in the various immune serums, varied between wide limits (cases 3282, 3332 and 3334).

4. Specific agglutinability of most of the strains was lost on prolonged cultivation (cases 3332 and 3370), but in some strains it was retained for a long time (cases 3334, 3365 and 3366).

In table 2 are summarized representative experiments with strains of the green-producing streptococcus isolated during life and after death in cases of influenzal pneumonia. The results with the strains isolated early in these cases were similar to those isolated in cases of influenza (cases 3206, 3207 and 3264), while late in the disease during life (cases 3265 and 3331), and after death (cases 3404, 3410, 3420 and 3436) the incidence of specific agglutination was decidedly lower, but even here the incidence was higher than that obtained with any of the other immune serums.

The agglutination experiments with the green-producing streptococcus isolated from the same patient throughout both the influenza attack and the influenzal pneumonia which followed showed that there was practically no difference in the immunologic condition of the strains isolated during influenza and during the early part of the influenzal pneumonia. Late in the pneumonic attack there was often a shifting of specific agglutination of these strains to one of the other serums, or agglutination to the same degree occurred in most of the immune serums; in other cases they might not be agglutinated by any of the serums. Thus in one case specific agglutination occurred in the serum of horse 15 of the primary culture from the sputum, and from the blood of a guinea-pig dead from an intraperitoneal injection of sputum, obtained on the third day of influenza. No agglutination occurred in any of the other serums. The colonies of the green-producing streptococci were quite moist and large, resembling type III pneumococci, but were not so mucoid in character, whereas on the

TABLE 2

AGGLUTINATION EXPERIMENTS WITH THE GREEN-PRODUCING STREPTOCOCCUS FROM INFLUENZAL PNEUMONIA

Case or Strain	Date of Experiment	Date of Isolation	Source	Day of Disease	Antiserums					Controls	
					Pneumococcus			Streptococcus			
					I	II	III	Hemolytic Horse 9	Green Producing Horse 15	Normal Horse Serum	NaCl Solution
3097	3/12/19	3/11/19	Sputum	1	0	++	0	0	0	0	0
3175.2	3/14/19	3/11/19	Sputum	4	++	+++	++	++	++++	+	0
3175[3].2	3/27/19	3/11/19	Sputum	4	0	0	0	0	++	0	0
3206	3/13/19	3/12/19	Sputum	2	0	0	0	0	+++	0	0
3207	3/13/19	3/12/19	Sputum	4	0	0	0	0	+++	0	0
3207[2].2	4/ 3/19	3/12/19	Sputum	4	0	++	0	0	+++	0	0
3264	3/25/19	3/24/19	Sputum	8	0	0	0	++	+++	0	0
3264	3/25/19	3/24/19	Sputum	8	0	0	0	++	+++	0	0
3264[2].2	3/28/19	3/24/19	Sputum	8	0	0	0	+	++	0	0
3265	3/25/19	3/24/19	Sputum	13	++	+	0	++	+++	+	0
3265.2	3/31/19	3/29/19	Sputum	18	0	0	++	0	0	0	0
3265.2	4/ 3/19	4/ 2/19	Sputum	21	0	0	0	0	0	0	0
3265[2].2	4/ 3/19	4/ 2/19	Sputum	21	0	0	0	0	0	0	0
3265.4	9/ 3/19	4/ 2/19	Sputum	21	+	++	+	++	+	+	0
3270	4/ 4/19	4/ 3/19	Sputum	12	0	0	0	+++	0	0	0
3270[2]	4/ 7/19	4/ 3/19	Sputum	12	0	0	0	++	0	0	0
3270.2	4/ 7/19	4/ 3/19	Sputum	12	0	0	0	++	0	0	0
3270[2].2	4/ 9/19	4/ 3/19	Sputum	12	0	0	0	++	++	0	0
3331	4/ 8/19	4/ 7/19	Sputum	2	0	0	++	++	+++	0	0
3331	4/ 8/19	4/ 7/19	Tonsil	2	0	0	0	0	++	0	0
3331	4/10/19	4/ 9/19	Sputum	4	0	0	0	0	0	0	0
3404	4/18/19	4/17/19	Throat	1	+	+	+	+	++	+	0
3404	4/21/19	4/20/19	Sputum	4	0	0	0	0	++	0	0
3404	4/21/19	4/20/19	Sputum	4	0	0		+	++	0	0
3404	4/25/19	4/24/19	Sputum	8	0	0	0	+++	0	0	0
3404	5/ 7/19	5/ 3/19	Lung after death	..	0	0	0	+	+	0	0
3404.2	5/ 8/19	5/ 6/19	Lung after death	..	0	0	0	++	++	0	0
2602.2	4/29/19	11/16/18	Lung after death	..	0	0	0	0	++	0	0
2630[2].2	4/29/19	11/30/18	Lung after death	..	+	+	+	+	+++	+	0
3228	3/19/19	3/18/19	Lung after death	..	0	0	0	++	+++	0	0
3287.3	4/ 8/19	3/31/19	Lung after death	..	0	0	0	0	+++	0	0
3287.3	4/ 8/19	3/31/19	Lung after death	..	0	0	0	0	+++	0	0
3410	4/21/19	4/19/19	Lung after death	..	0	0	0	0	0	0	0
3415	4/21/19	4/19/19	Lung after death	..	0	0	0	0	+++	0	0
3420	4/24/19	4/22/19	Lung after death	..	0	0	0	0	0	0	0
3433	4/25/19	4/24/19	Lung after death	..	++++	0	0	0	0	0	0
3436	4/26/19	4/25/19	Lung after death	..	0	0	0	+++	0	0	0

second and sixth days of the pneumonia which followed, specific agglutination occurred in type III pneumococcus serum of the primary culture from the sputum in four tests. Less agglutination occurred in the serum of horse 15 in three instances, and in two instances in each of the type II pneumococcus serum and hemolytic streptococcus serum

of horse 9. In another case of typical influenza the green-producing streptococcus in the primary culture and in the first subculture from the sputum on the third day was agglutinated specifically by the serum of horse 9, while on the second day of the influenzal pneumonia which followed, and after death, it was not agglutinated by any of the serums. In still another case specific agglutination occurred in the serum of horse 15 during influenza and early in influenzal pneumonia, whereas

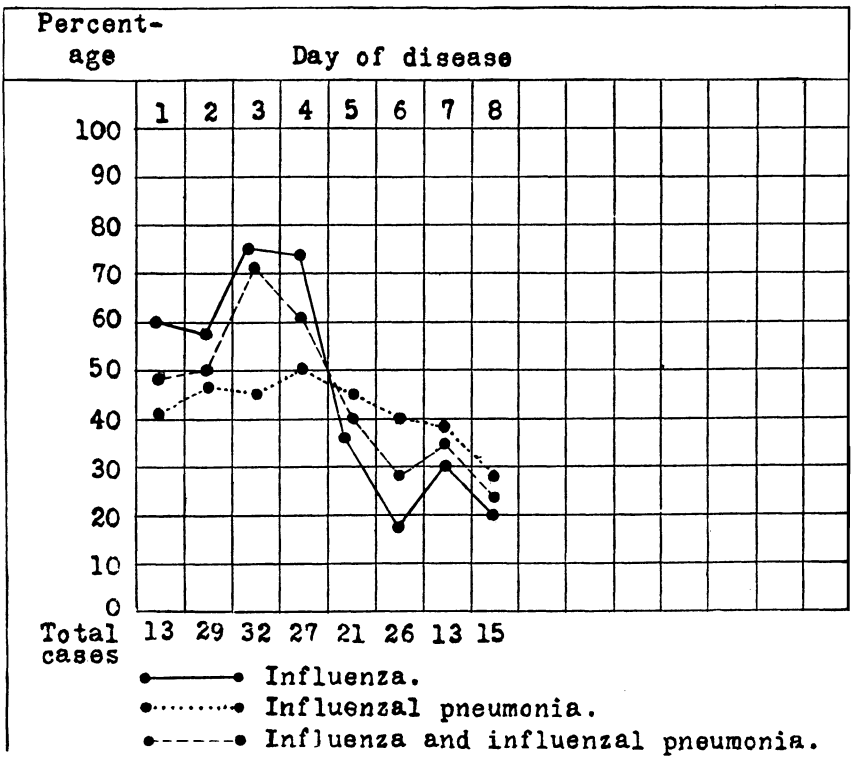

Chart 1.—Percentage of specific agglutination in the monovalent serum of the green-producing streptococcus from influenza and influenzal pneumonia according to the day of the disease.

later in the pneumonic attack specific and marked agglutination occurred in type II pneumococcus serum and lesser agglutination in the serum of horse 15.

In chart 1 is given graphically the average percentage incidence of specific agglutination by the monovalent serum of the green-producing streptococci from influenza and influenzal pneumonia, according to the

days of the disease. The curves represent the results obtained on the days indicated. The antigen consisted throughout of the primary culture of the sputum in dextrose-blood broth or of animals injected with sputum irrespective of what the culture showed on plating, and with the early subcultures containing the green-producing streptococcus. The close parallelism between the strains isolated in influenza without frank signs of lung involvement and the cases of influenzal pneumonia is shown by the fact that the average incidence of specific agglutination, while somewhat lower in the latter, runs roughly parallel. The average incidence of specific agglutination for both influenza and influenzal pneumonia strains was highest during the first four days, when a gradual decline occurred up to and including the eighth day. A number of facts indicate that these strains of different agglutinability which appear late in the pneumonic attack are modifications of the strains which are agglutinated specifically by the monovalent serum early in the attacks, and that their appearance is not always the result of superimposed infections from without. The specific strains tend to lose this property on artificial cultivation. The various strains have been found to be unstable in their cultural character and fermentative reactions.

There was no parallelism between the occurrence of specific agglutination in the serum of horse 15 of the different strains and their power to ferment inulin, or their solubility in bile.

Moreover, marked changes in the immunologic condition as measured by agglutination tests have occurred in a number of strains following successive (intratracheal) animal passages.

Thus strain 2719 was agglutinated completely and specifically by the serum of horse 15, as isolated and after one animal passage. Less agglutination occurred in the serum of horse 9, but none in any of the other serums, whereas after the third and fourth animal passages agglutination in the serum of horse 15, while still specific, was less marked and some agglutination occurred in each of the other serums.

In strain 2749 a similar change in agglutinability occurred during the third and fourth animal passages.

In case 2800 the patient from whom the strain was isolated with which horse 15 was immunized, specific agglutination increased during the first and second animal passages over that noted before animal passage, and a marked diminution in agglutination occurred in the serum of horse 15 after the fourth animal passage.

In summarizing the agglutination tests which were made in a routine manner throughout the epidemic of 1918-1919 it was found that material from influenza and influenzal pneumonia, without regard to the time in the attack when the sputum or other material was obtained for culture, and without regard to the type of flora the cultures of the sputum or primary culture showed, was subjected to the agglutinating action of the monovalent immune serum in 567 experiments, representing 184 cases. Of these, 295 (52 per cent.) showed specific agglutination in the serum of horse 15. The primary culture of the sputum in many instances, especially late in the disease, and of the lung exudate after death, showed predominating numbers of hemolytic streptococci, less often staphylococci and rarely colon bacilli or bacillus mucosus.

Specific agglutination occurred in the serum of horse 15 in 29 instances (58 per cent.) of 50 experiments, representing 25 cases, and in 20 of the 25 cases in which the antigen consisted of a salt solution suspension of the primary culture of the sputum or blood of animals dead from injection of sputum, or of pure cultures of these after suspension in 50 per cent. glycerol for some months. All these were cultures from cases which occurred during the first two waves of the disease.

The relative significance of these figures becomes more apparent from a study of tables 1 and 2, in which it is shown that there is a relatively greater frequency and a greater degree of agglutination in the serum of horse 15 over those in the other immune serums; that the antihemolytic streptococcus serum (horse 9) ranks second, and that with few exceptions only a slight difference occurred between the type pneumococcus serums and normal horse serum. The exact figures of the total average incidence of agglutination from slight to marked agglutination in the different serums of the strains from influenza and influenzal pneumonia were found to be as follows: type I pneumococcus serum in 20 per cent. of 563 tests; type II pneumococcus serum in 22 per cent. of 525 tests; type III pneumococcus serum in 21 per cent. of 524 tests; horse 9 serum in 39 per cent. of 561 tests; horse 15 serum in 61 per cent. of 567 tests; normal horse serum in 23 per cent. of 556 tests; salt solution in 7 per cent. of 555 tests. It is certain that the high incidence of agglutination in horse 15 serum was not due to nonspecific effects, since its agglutinating power over 72 strains of green-producing streptococci or pneumococci from sources other than influenza was 25 per cent., or about that of normal horse serum. Moreover, the average amount of agglutination in the serum of horse 15 with the

influenza strains was much higher than in the other serums. The control strains included, in addition to type pneumococci and hemolytic streptococci, green-producing streptococci from a wide range of sources, such as the nose and throat of normal persons, the nose of normal guinea-pigs, throats in simple nasopharyngitis, the central nervous system in poliomyelitis, ulcer of the stomach, and arthritis.

As I have pointed out, there was a tendency of the green-producing streptococci to become heterogeneous and to lose the property of specific agglutination after prolonged cultivation on artificial mediums. This varied greatly with different strains (tables 1 and 2). One hundred and fourteen strains after cultivation on artificial mediums (chiefly blood agar) for from 6 to 10 months were subjected to the agglutinating action of the monovalent and the other serums. In these only 26 strains, or 23 per cent., were agglutinated specifically by the monovalent serum. This low figure was no doubt due in part to the deterioration of the serum. It has been pointed out elsewhere (study III) that as these strains are cultivated on artificial mediums they tend to agglutinate spontaneously in liquid cultures, and many strains are unsuited for agglutination tests. This tendency was noted also in the strains which grew diffusely in that the incidence of nonspecific agglutination in the various serums was considerably higher than in the freshly isolated strains. Thus of the 114 experiments, nonspecific agglutination, usually slight, occurred in type I pneumococcus serum in 25 per cent.; type II, in 23 per cent.; type III, in 24 per cent.; antihemolytic streptococcus serum horse 9, in 54 per cent.; monovalent serum horse 15, in 78 per cent., and in normal horse serum, in 35 per cent.

The close relationship between the green-producing streptococcus and hemolytic streptococcus in influenza is shown by the fact that 18 per cent. of 44 strains of hemolytic streptococci isolated during life and after death in influenza were agglutinated specifically by the serum of horse 15.

Beside the time in the attack in which the cultures were made (chart 1) and the predominating flora at hand, the instability of the strains of green-producing streptococci had to be taken into consideration in properly interpreting the results of the agglutination experiments, for by plating the culture actually agglutinated it was found that nonspecific agglutination by the serum of horse 15 was often due either to the fact that green-producing streptococci were not inoculated, or marked changes had occurred in the culture. After these discrepan-

cies, and the earlier experiments in which plates were not made of the cultures actually agglutinated are eliminated, there are in all 252 tests in which the culture subjected to the agglutinating action of the serum was proved to contain green-producing streptococci. Of the 252 tests, 120, representing 92 different cases, were made with the green-producing streptococci in the primary culture of dextrose-blood or acacia broth from sputum and throat exudate during life and lung exudate after death. In 72 (60 per cent.) specific agglutination occurred in the serum of horse 15. Of 27 tests, representing 16 cases, 19 (70 per cent.) showed specific agglutination in this serum in the primary culture of blood or peritoneal exudate of animals dead from injection of sputum, or primary culture of sputum. In the remaining 105 tests in which pure cultures of the green-producing streptococci in from the first to the sixth subcultures were used as antigen, representing 90 cases of influenza or influenzal pneumonia, 85 (81 per cent.) showed specific agglutination in the monovalent serum. Thus specific agglutination of the green-producing streptococci, which was proved to be contained in the antigen used, occurred in the monovalent serum in 176 of 252 agglutination experiments, an average of 70 per cent. Hence this figure may be taken to express roughly the percentage of the strains of green-producing streptococci which were immunologically identical and found throughout influenza and influenzal pneumonia.

Through the kindness of Major Fennell, of the Army Medical School, I have had an opportunity to test the behavior of strains of green-producing streptococci and type IV pneumococci, which he obtained from widely separated localities, toward the monovalent serum of horse 15. The source of these strains and their immunologic condition as measured by the various immune serums are given in table 3. Specific agglutination was obtained in 12 of 16 strains, or in 75 per cent. of the strains isolated from influenzal pneumonia, and in no instance in four other strains, one isolated from the normal mouth in Washington during the epidemic and three strains of pneumococci which Major Fennel isolated from spontaneous pneumonia in the monkey. A study of the results obtained with these strains in relation to their solubility in bile and their ability to ferment inulin shows that in these strains, as in those isolated in Rochester, specific agglutination does not depend either on whether they are or are not bile soluble, or whether they do or do not ferment inulin. Some of the negative agglutinations may be due to the fact that the strains had been culti-

vated for some time before the agglutination tests were made, all being in at least the eighth subculture. The incidence of agglutination of these strains by the other serums is about that of the strains isolated by us.

TABLE 3

AGGLUTINATION EXPERIMENTS WITH STRAINS OF GREEN-PRODUCING STREPTOCOCCI FROM WIDELY DISTANT LOCALITIES

Strain	Source	Antiserums					Controls		Solubility in Bile	Acid in Inulin
		Pneumococcus			Streptococcus					
		I	II	III	Hemolytic Horse 9	Green Producing Horse 15	Normal Horse Serum	NaCl Solution		
S 1	Influenzal pneumonia, Camp Wheeler	0	0	+	+	++++	0	0	0	+
S 3	Influenzal pneumonia, Camp Wheeler	0	0	0	0	++++	0	0	0	0
S 3	Influenzal pneumonia, Camp Wheeler	0	0	0	0	++++	0	0	0	0
S 5	Influenzal pneumonia, Camp Wheeler	0	+++	0	++	++++	0	0	0	0
S 6	Influenzal pneumonia, Camp Wheeler	0	0	0	0	++	0	0	0	0
55	Influenzal pneumonia, Camp Wheeler	0	0	0	0	+	0	0	+	+
S 14	Influenzal pneumonia, Chicago	0	0	0	0	+++	0	0	0	0
S 24	Influenzal pneumonia, Chicago	0	0	0	++	+++	0	0	0	0
S 17	Influenzal pneumonia, Walter Reed Hospital	0	0	0	0	0	0	0	0	0
24	Influenzal pneumonia, Walter Reed Hospital	0	0	0	++	+++	0	0	+	+
15	Influenzal pneumonia, Camp Sherman	0	0	0	0	++	0	0	+	+
S 19	Influenzal pneumonia, Camp Sherman	0	0	0	+++	++++	+	0	0	+
S 25	Influenzal pneumonia, Camp Sherman	0	0	0	0	0	0	0	0	0
S 27	Influenzal pneumonia, Johns Hopkins	0	0	0	0	0	0	0	0	0
113	Influenzal pneumonia, Camp Greene	0	0	0	0	+++	0	0	+	+
152	Influenzal pneumonia, Camp Greene	0	0	0	0	0	0	0	+	+
149	Normal mouth, Washington during epidemic	0	0	0	0	0	0	0	+	+
194	Spontaneous pneumonia, monkey	0	0	0	0	0	0	0	+	+
212	Spontaneous pneumonia, monkey	0	0	0	0	0	0	0	+	+
225	Spontaneous pneumonia, monkey	0	0	0	0	0	0	0	+	+
225	Spontaneous pneumonia, monkey	0	0	0	0	0	0	0	+	+

Owing to the instability of the green-producing streptococci from influenza, and the tendency to the development of mutation forms, it was found necessary to inoculate a blood-agar plate with the culture subjected to the agglutinating action of the different serums in order to interpret properly the results obtained.

Striking examples of the development of mutation forms as measured by changes in morphology in cultural characteristics and in immunologic conditions were noted in many instances. The source of the micro-organism, the culture medium inoculated and the effect of the various serums in some of the cultures which yielded mutation forms on plating are summarized in table 4. It will be noted that in the first nine experiments, hemolytic streptococci were obtained on the blood-agar plate in pure culture in eight and together with staphylococci in two when single colonies or groups of well isolated colonies of green-producing streptococci were inoculated, and that specific agglutination by the serum of horse 9 occurred in three, and in the serum of horse 15 in one instance. The rest were not agglutinated by any of the serums.

In experiments 10 to 13, inclusive, pure cultures of green-producing streptococci were obtained from the dextrose-blood or dextrose-acacia broth when single colonies of hemolytic streptococci were inoculated. One of these was agglutinated specifically by the hemolytic streptococcus, the other by the green-producing streptococcus antiserums. In these experiments it is assumed that the culture actually agglutinated contained the type of streptococcus homologous to the serum which agglutinated specifically. Proof that this was actually the case could not be obtained, because it is conceivable that mutation forms might develop not in the broth culture, but as growth occurred on the blood-agar plate, and the morphology of the two types of streptococci were so similar that differentiation in this way was not possible.

In experiments 14 to 24, inclusive, in which the mutant was a streptococcus, the morphology and immunologic condition were sufficiently different from that of the organisms inoculated to make it possible to determine where the mutation occurred. Specific or marked agglutination in the monovalent serum occurred in the dextrose-blood or acacia broth cultures in all but one of these strains. Smears of the cultures agglutinated in these showed no staphylococci, but typical elongated diplococci singly or in chains of variable length.

Smears of those in which agglutination did not occur (experiment 24) showed staphylococci, and agglutination experiments with streptococci from the sputum in influenza were not agglutinated by this or other serums (experiments 29 to 34). The number of staphylococcus colonies on the plates was often very large. Their number and distribution on the plates were such as to exclude the possibility of contamination from the air. Hence it is certain that mutation must have occurred on the blood-agar plates, and that many of the organisms in

TABLE 4

THE RELATION OF THE AGGLUTINABILITY OF BACTERIA FROM INFLUENZA TO THE DEVELOPMENT OF MUTATION FORMS

Number	Strain	Micro-organism Inoculated	Culture Used in Agglutination Test	Antiserums					Controls		Growth on Blood-Agar Plate of Culture Agglutinated
				Pneumococcus			Streptococcus				
				I	II	III	Hemolytic Horse 9	Green Producing Horse 15	Normal Horse Serum	NaCl Solution	
1	2698[2]	Green-producing streptococcus from pleural fluid of guinea-pig injected with sputum	Dextrose-blood broth	0	0	0	+++	++	0	0	Hemolytic streptococci and staphylococci
2	3083.4	Single colony of green-producing streptococcus from sputum	Dextrose-blood broth	0	0	0	0	0	0	0	Slightly hemolytic streptococci
3	3270.2	Single colony of green-producing streptococcus from sputum	Dextrose-blood broth	0	0	0	0	0	0	0	Hemolytic streptococci and staphylococci
4	3296[2]	Green-producing streptococcus from blood of mouse injected with sputum	Dextrose-blood broth	0	0	0	0	0	0	0	Hemolytic streptococci
5	3297.2	Single colony of green-producing streptococcus from sputum	Dextrose-blood broth	0	0	0	0	0	0	0	Hemolytic streptococci
6	3300.2	Group of green-producing streptococcus colonies from throat	Dextrose-blood broth	0	0	0	0	0	0	0	Hemolytic streptococci
7	3358	Green-producing streptococcus from sputum	Dextrose-acacia broth	0	0	0	++++	0	0	0	Hemolytic streptococci
8	3394[2].2	Single colony of green-producing streptococcus from blood of mouse injected with sputum	Dextrose-acacia broth	0	0	0	0	++	0	0	Hemolytic streptococci
9	3398.5	Single colony of green-producing streptococcus from sputum	Dextrose-blood broth	0	0	0	++	0	+	0	Hemolytic streptococci
10	3266[2].10	Single colony of slightly hemolytic streptococcus	Dextrose-blood broth	0	0	0	0	0	0	0	Green-producing streptococci
11	3048.3	Single colony of green-producing streptococcus from hemolytic streptococcus	Dextrose-acacia broth	0	0	0	++	+	0	0	Green-producing streptococci
12	3387[2].5	Slightly hemolytic streptococcus.....	Dextrose-blood broth	0	0	0	0	0	0	0	Green-producing streptococci
13	2698[2].2	Single colony of hemolytic streptococcus....	Dextrose-blood broth	0	0	0	0	+++	0	0	Green-producing streptococci
14	2719	Green-producing streptococcus from pleural fluid of guinea-pig injected with sputum	Dextrose-blood broth	0	0	0	++	+++	0	0	Staphylococci
15	2719[4].4	Single colony of green-producing streptococcus	Dextrose-blood broth	+	+	+	+	++	0	0	Staphylococci
16	2757[2].4	Green-producing streptococcus from blood of guinea-pig injected with sputum	Dextrose-blood broth	0	0	0	++	+++	+	0	Staphylococci
17	2800[2]	Green-producing streptococcus from blood of guinea-pig injected with sputum	Dextrose-blood broth	0	0	0	++	+++	+	0	Staphylococci
18	3225[2].3	Green-producing streptococcus from blood of mouse injected with sputum	Dextrose-blood broth	0	0	0	0	+	0	0	Staphylococci
19	3241[2]	Green-producing streptococcus from blood of guinea-pig injected with sputum	Dextrose-acacia broth	0	++	0	0	++++	0	0	Staphylococci

20	3241[2].2	Single colony of green-producing streptococcus from blood of guinea-pig injected with sputum	Dextrose-acacia broth	0	0	0	++	+++	0	0	Staphylococci
21	3270[2].2	Green-producing streptococcus from blood of guinea-pig injected with sputum	Dextrose-acacia broth	0	0	0	++	++	0	0	Staphylococci
22	3296[2].2	Green-producing streptococcus from blood of mouse injected with sputum	Dextrose-acacia broth	0	0	0	++	++	0	0	Staphylococci
23	3334.2	Single colony of green-producing streptococcus from sputum	Dextrose-acacia broth	0	0	+++	0	+++	0	0	Staphylococci
24	3434[2]	Green-producing streptococcus from blood of mouse injected with sputum	Dextrose-blood broth	0	0	0	0	0	0	0	Staphylococci
25	2917.3	Single colony of hemolytic streptococcus.....	Dextrose-acacia broth	0	0	0	0	0	0	0	Staphylococci
26	3211.2	Single colony of hemolytic streptococcus.....	Dextrose-acacia broth	0	0	0	0	0	0	0	Staphylococci
27	3296[2]	Single colony of hemolytic streptococcus.....	Dextrose-acacia broth	0	0	0	++	++	0	0	Staphylococci
28	3334.2	Single colony of slightly hemolytic streptococcus	Dextrose-acacia broth	0	0	0	++	++	0	0	Staphylococci
29	2608[3].2	Single colony of staphylococcus from sputum	Dextrose-blood broth	0	0	0	0	0	0	0	Staphylococci
30	3175.4	Single colony of staphylococcus from sputum	Dextrose-blood broth	0	0	0	0	0	0	0	Staphylococci
31	3208.2	Single colony of staphylococcus from sputum	Dextrose-blood broth	0	0	0	0	0	0	0	Staphylococci
32	3302.2	Staphylococcus from sputum.....	Dextrose-blood broth	0	0	0	0	0	0	0	Staphylococci
33	3357.2	Single colony of staphylococcus from sputum	Dextrose-acacia broth	0	0	0	0	0	0	0	Staphylococci
34	3434.2	Staphylococcus from sputum.....	Dextrose-blood broth	0	0	0	0	0	0	0	Staphylococci
35	3334.2	Single colony of influenza bacillus from sputum	Dextrose-blood broth	0	0	+	0	++	0	0	Green-producing streptococci
36	3334.2	Single colony of influenza bacillus from sputum	Dextrose-blood broth	0	0	0	+++	++++	0	0	Green-producing streptococci
37	3341.2	Single colony of influenza bacillus from sputum	Dextrose-blood broth	0	0	0	0	0	0	0	Green-producing streptococci
38	3332.2	Single colony of influenza bacillus from sputum	Dextrose-blood broth	0	0	0	0	+	0	0	Slightly hemolytic streptococci
39	3332.2	Single colony of influenza bacillus from sputum	Dextrose-blood broth	0	0		0	0	0	0	Hemolytic streptococci
40	3248.2	Single colony of influenza bacilus from sputum	Dextrose-blood broth	0	0	0	0	0	0	0	Moist hemolytic streptococci
41	3283.8	Single colony of influenza bacillus from evaporated oil suspension	Dextrose-blood broth	0	+++	0	0	++	0	0	Green-producing streptococci and staphylococci
42	3201.3	Single colony (twice) of influenza bacillus from sputum	Dextrose-blood broth	0	+++	0	0	++	0	0	Staphylococci
43	3178.8	Group of colonies of influenza bacillus from sputum	Dextrose-blood broth	0	0	0	0	0	0	0	Bacillus influenza
44	3353[2]	Influenza bacilli from blood of mouse.....	Dextrose-blood broth	0	0	0	0	0	0	0	Bacillus influenza

the tall cultures of dextrose-blood or acacia broth took part in this process. The lack of specific agglutination when the cultures of hemolytic streptococci yielded staphylococci is also in harmony with this idea.

Similar results were obtained in many instances in which influenza bacilli were inoculated into the dextrose-blood broth (experiments 35 to 44). In most of these the culture of influenza bacillus was derived from a single colony on blood-agar plates inoculated with sputum, and in these it is conceivable, but not probable, that what appeared as a mutation might merely be the growth of this organism in the broth when inhibited on the blood-agar plate, the contact on the blood-agar plate inoculated with the sputum not being sufficiently intimate to allow growth of one or a few organisms.

Control inoculations from the colony fished in these as well as in the streptococcus experiments made in the immediate neighborhood of the colony or on another blood-agar plate showed only the growth characteristic of the colony from which inoculated. Moreover, similar results were obtained with some strains after many subcultures and after repeated platings from single colonies (experiments 41 and 42). The mode of occurrence, the immunologic condition, the control cultures of the blood used in the broth, and finally, the fact that the mutants were often highly virulent, rule out all reasonable possibility that we were dealing with contaminations, but, as pointed out elsewhere, final conclusions cannot be drawn until the pure line requirement has been fulfilled.

The suddenness and degree of the changes noted throughout these studies were similar to those I noted in a study on the transmutation of pneumococci and streptococci, and to those described by Clough in a study of pneumococci reacting with all of the three antipneumococcus type serums and in which a striking example of mutation occurred.

By the use of various immune serums, including the monovalent serum, it may be concluded that the somewhat peculiar green-producing streptococci noted at the outset of the epidemic and isolated so constantly since, both in influenza and influenzal pneumonia, are immunologically quite homogeneous. A high percentage of the strains, especially those isolated early in the attacks, are agglutinated specifically in the serum prepared with one of these strains. Highly agglutinable strains, as has been shown, absorb the agglutinins for other strains. The serum of patients recovering from influenza acquires agglutinating power over homologous and other strains. This finding

is in accord with those of Tunnicliff and of Howell and Anderson, who also find immunologic evidence of the identity of green-producing streptococci from influenza. Specific agglutination occurred in the monovalent serum irrespective of whether or not they fermented inulin or of whether they were bile soluble or insoluble. After cultivation on artificial mediums, and after repeated animal passages, as well as late in influenza and influenzal pneumonia, the strains tend to become more heterogeneous.

The findings of immunologically dissimilar green-producing streptococci late in influenza is in harmony with the results obtained by Mathers in a study of pneumococci in reinfection in lobar pneumonia in which the type was also found to change. Evidence has been obtained to show that the mutation forms which develop in vitro and in vivo in animals are in general similar immunologically to the organisms commonly isolated in influenza. It has been shown elsewhere that they resemble these also in infecting power. Hence, it would seem that mutation may play an important rôle in the pathogenesis of influenza.

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